Remarks

Claims 7-9 and 31-38 are pending in the application. Claims 31 and 36-38 have been amended and new claim 39 has been added. Support for the amendments may be found throughout the specification, including the claims as originally filed. No new matter has been added.

Amendment of claims should in no way be construed as an acquiescence to any of the Examiner's rejections. The amendments to the claims are being made solely to expedite prosecution of the present application. Applicants reserve the option to further prosecute the same or similar claims in the instant or in a subsequent patent application.

Applicants respectfully request entry of these claim amendments, since these amendments present the claims in better form for consideration on appeal.

Rejection of claims 7-9 and 31-38 under 35 U.S.C 101 and 35 U.S.C. 112, first paragraph

The Examiner states that claims 7-9 and 31-38 stand rejected under 35 U.S.C. 101 because the claimed invention is not supported by either a specific and substantial, credible asserted utility or a well established utility. The Examiner further states that claims 7-9 and 31-38 stand rejected under 35 U.S.C. 112, first paragraph because if the invention is not supported by a specific and substantial, credible asserted utility or a well established utility one skilled in the art would not know how to use the claimed invention. Applicants respectfully traverse the rejection.

Examiner states that Applicant's argument "presupposes that the genes identified by the method will themselves have a substantial utility. Yet the specification and claims are not directed to any particular genes which are capable of being identified which themselves have been shown to possess substantial utility." Furthermore, according to the Examiner, "the instant assay fails to correlate any particular gene product with any particular disease, but merely contemplates that such could be discovered by screening for players which are up- or down-regulated in a differentiating model system."

Applicants respectfully submit that §2107.01 of the MPEP, General Principles Governing Utility Rejections, state that a "'substantial utility' defines a 'real world' use." The MPEP further

states that "an assay method for identifying compounds that themselves have a "substantial utility" define a "real world" context of use." As described in the specification dedifferentiation of smooth muscle cells results in numerous diseases, e.g., occlusive arteriosclerotic diseases, atherosclerosis, heart attacks, strokes, restenosis, and hypertension (paragraph bridging pages 1 and 2; and page 45, lines 9-23). Accordingly, genes that are up- or down-regulated during differentiation of neural crest cells into smooth muscle cells can be used as markers of the stage of differentiation of these cells, and thus, as diagnostic markers, for predicting whether a subject has an increased probability of developing, e.g., an occlusive arteriosclerotic disease. The Examiner states that "[w]hile, e.g. an occlusive arteriosclerotic disease is contemplated by the specification as being a disease which would likely be associated with genes which were up- or down- regulated as claimed, no correlation between any such particular gene product and occlusive arteriosclerotic disease in patients has been disclosed." Applicants respectfully point out that identification of a specific gene product correlated with, e.g. arteriosclerosis, is not necessary as the detection of the presence of differentiated or undifferentiated cells alone would be indicative of the disease or lack thereof. Importantly, the present invention teaches a person skilled in the art to screen for genes that are correlated with a particular differentiation state. Since the presence or absence of a gene product would be indicative of the differentiation state of the cell and thus the likelihood of developing, e.g. arteriosclerosis, the utility of the invention is readily apparent to one skilled in the art. Furthermore, the present invention does not "require or constitute carrying out further research to identify or reasonably confirm a 'real world' context of use."

The Examiner states that the "instant assay fails to correlate any particular gene product with any particular disease, but merely contemplates that such could be discovered by screening for players which are up- or down- regulated in a differentiating model system." Furthermore, the Examiner states that the "claimed invention does not provide an identified gene which is up- or down- regulated, diagnostically correlated or useful to determine disease incidence, likelihood or differentiation state...."

Applicants respectfully point out that the application does disclose the identification of a novel gene product that is correlated with the differentiation state of the cell. In particular, Example 2 discloses that aortic carboxypeptidase-like protein (ACLP) is not expressed in neural crest cells, but is up-regulated in differentiated smooth muscle cells (see page 53, lines 32-33 and

54, lines 1-3 and lines 18-20 and page 60, lines 11-13). The absence of ACLP in vascular smooth muscle would therefore be indicative of cellular dedifferentiation and a predisposition to, e.g., arteriosclerotic disease.

Additionally, a recent publication listing the inventors as authors further underscores the specific and substantial utility of the invention. In Watanabe, et al. (2002) Circulation Research, 91(5):382-9, the inventors disclose the identification of a novel regulator of smooth muscle differentiation and proliferation, modular recognition factor 2 (Mrf2), using the methods disclosed in the present application. Mrf2 expression was induced by differentiation into smooth muscle cells and is specifically correlated with that differentiation state. Furthermore, Watanabe et al., provides experimental evidence that overexpression of Mrf2 retarded cellular proliferation. The ability to measure the expression level of Mrf2 allows one skilled in the art to readily ascertain the differentiation state of the cells and therefore the likelihood of developing, e.g., arteriosclerotic disease. Thus, the claimed assay method is useful for identifying genes which themselves have a substantial utility and, therefore, define a real world use of the invention. A copy of Watanabe et al., is attached hereto as Exhibit A.

Thus, reconsideration and withdrawal of the rejection of claims 7-9 and 31-38 under 35 U.S.C. 101 is respectfully requested.

The Examiner also reiterates her objection that claims 7-9 and 31-38 stand rejected under 35 U.S.C. 112, first paragraph on the basis that if the invention is not supported by a specific and substantial, credible asserted utility or a well established utility one skilled in the art would not know how to use the claimed invention. Applicants respectfully submit that in light of the specific and substantial utility of the invention as outlined above, one skilled in the art would readily know how to use the claimed invention. Thus, reconsideration and withdrawal of the rejection of claims 7-9 and 31-38 under 35 U.S.C. 112, first paragraph is respectfully requested.

Rejection of claims 31 and 36-38 under 35 U.S.C. 112, second paragraph

The Examiner states that claims 31 and 36-38 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to point out and distinctly claim the subject matter which applicant regards as the invention. Claims 31 and 36-38 have been amended to obviate the Examiner's rejection.

Additionally, Applicants have inserted new claim 39 which makes reference to Table I. The Examiner objected to such language on the basis that the claim "appears to attempt incorporation of certain elements as recited in a Table of the specification at p. 14. While the claims are read in light of the specification, the specification cannot be read into the claims. Thus, where applicant's wish to limit the elements of the culture conditions such limitations are required to be recited in the claims. The artisan cannot discern the metes and bounds of the claim." Applicants respectfully point out that §2173.05(s) of the MPEP permits incorporation by reference of subject matter in a figure or table into a claim "where it is more concise to incorporate by reference" as compared to "duplicating a drawing or table into the claim."

Accordingly, reconsideration and withdrawal of the rejections of claims 31 and 36-38 under 35 U.S.C. §112, second paragraph, is respect requested.

Rejection of claims 7, 32, 34 and 35 under 35 U.S.C. 102(e) as being anticipated by Anderson et al., U.S. Patents 5,672,499 and 6,001,654

The Examiner states that claims 7, 32, 34 and 35 stand rejected under 35 U.S.C. 102(e) as being anticipated by Anderson et al., US Patent No. 5,672,499 filed June 7, 1995, issued September 30, 1997 and as anticipated by Anderson et al., US Patent No. 6,001,654 filed April 25, 1997, issued Dec. 14, 1999. Applicants respectfully traverse these rejections.

Applicants respectfully resubmit that "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987). Neither US Patent '499, nor US Patent '654, set forth each and every element of the present invention.

Applicants again point out that, contrary to the Examiner's statement, Applicants have not acknowledged that the '654 and '499 references teach examples of immortalized neural crest cells. As pointed out in the last submitted Office Action response, at most, Applicants merely indicated that "the references contain a prophetic example indicating how one could immortalize neural crest stem cells," (emphasis added).

Applicants maintain that the '499 and '654 references fail to teach differentiation of immortalized neural crest cells into smooth muscle cells. The Examiner directs the Applicants to column 19, lines 27-55, column 31, line 45 and column 32, line 25 of the '654 patent for disclosure regarding the immortalization of neural crest cells via v-myc transformation as claimed in the present invention. However, the recited portions of '654 only contemplate prophetic methods of immortalizing neural crest cells. The '654 invention is solely directed to methods of culturing and differentiation of primary neural crest cells. Specifically, column 16, lines 60-63, explicitly states that the invention is directed to differentiation of smooth muscle cells in vitro from a "naturally occurring precursor."

Furthermore, the Examiner acknowledges on p.9 of the Office Action that "Anderson et al., does not teach the method of claim 7, wherein the cells are Monc-1 cells." The specification, page 49, lines 30-32, explicitly point out that Monc-1 cells are an immortalized cell line generated by retroviral transfection of mouse neural crest cells with the v-myc gene. Applicants respectfully point out that the Examiner has acknowledged that the Anderson et al reference does not teach the method of claim 7, wherein the cells are immortalized.

The Examiner further states that the methods disclosed by Anderson et al. in the '654 patent result in "differentiation of smooth muscle cells and the identification of the gene alpha smooth muscle actin, peripherin, and calponin which are up regulated upon differentiation in culture to smooth muscle cells, see for example Figure 20, 22, claims 6 and 21" (see Office Action at page 6). However, in contrast to the assertions in the Office Action, Anderson et al. do **not** teach a *method for identifying a gene* which is up- or down- regulated during the differentiation of neural crest cells into smooth muscle cells. Rather, Anderson et al., merely use known smooth muscle cell markers to establish the presence of smooth muscle cells in their cell culture. Applicants respectfully direct the Examiner to column 33 of the '654 patent, lines 26-31 and lines 58-67 and column 34, lines 1-6. In contrast to the invention as claimed in the present application, neither the '499 nor the '654 patent teach an assay method for identifying a gene which is up- or down- regulated during the differentiation of neural crest cells into smooth muscle cells. Therefore neither of the Anderson et al. references anticipates the method as claimed in the present invention. Additionally, the cells contemplated in the '654 patent are not immortalized.

A claim is anticipated only if each and every element of the claim is found in a single prior art reference. Neither the '499 or the '654 patent teach each and every element of the claims in the present application. Therefore, reconsideration and withdrawal of the rejection of claims under 35 U.S.C. 102(e) is respectfully requested.

Rejection of claims 33 under 35 U.S.C. 103(a)

Claim 33 is rejected under 35 U.S.C. 103(a) as being unpatentable over Anderson et al., US Patent 5,672,499, Anderson et al., US Patent 6,001,654, Rao et al., (1997) J. of Neurobiology, 32: 722-746, and Sommer et al., (1995) Neuron, 15:1245-58. Applicants respectfully traverse the rejection.

The Examiner relies on Anderson et al., US Patent 5,672,499 and Anderson et al., US Patent 6,001,654 for the teaching of methods 7, 32, 34 and 35, "including the suitability of the method for identifying up and down regulation of gene expression in neural crest cells using neural crest cell lines transformed with v-myc" (Office Action at page 9). The Examiner further relies on Rao et al. and Sommer et al., for the teaching that neural crest cells may be immortalized via the transformation with v-myc and that such cells are termed Monc-1 cells. The Examiner states that "one skilled in the art would have been motivated to substitute the Monc-1 cells of Rao and Sommer in the method of Anderson, based on Anderson's teachings of the suitability of such v-myc transformed neural crest cells in the study of up and down regulation of gene expression during differentiation to smooth muscle cells."
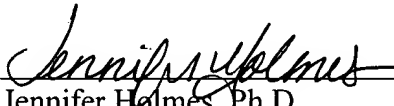
As discussed above, Applicants respectfully submit that Anderson et al. (U.S. Patent Nos. '499 and '654) fail to teach *a method for identifying genes* that are either up- or down- regulated during the differentiation of neural crest cells into smooth muscle cells. Furthermore, the Rao et al. and Sommer et al. references do not make up for the deficiencies of the teachings of Anderson et al. (U.S. Patent Nos. '499 and '654) as they also fail to teach a method for identifying genes that are either up- or down- regulated during the differentiation of neural crest cells into smooth muscle cells. Therefore, the cited references, either alone or in combination, fail to teach or suggest the invention of claim 33.

Thus, reconsideration and withdrawal of the rejection of claim 33 under 103(a) is respectfully requested.

Conclusion

In view of the above remarks and the amendments to the claims, it is believed that this application is in condition for allowance. If a telephone conversation with Applicant's Attorney would expedite prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 832-1000.

Respectfully submitted,

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Copy of amended claims with changes marked thereon

Please amend claims 31 and 36-38 as set forth below:

31.   **(Twice Amended)** The method of claim 7, wherein the culture conditions [include the medium identified in Table 1] comprise a smooth muscle cell differentiation medium.

36.   **(Amended)** The method of claim 31, wherein the [culture conditions further include] smooth muscle cell differentiation medium comprises 10% fetal bovine serum [and 25 mM Hepes (pH 7.4].

37.   **(Amended)** The method of claim 31, wherein the [culture conditions] smooth muscle cell differentiation medium [do] does not include exogenously added active TGF-β1.

38.   **(Amended)** The method of claim 31, wherein the [culture conditions] smooth muscle cell differentiation medium [further include] comprises exogenously added active TGF-β1.

Please insert a new claim 39:

39.   **(New)** The method of claim 31, wherein the smooth muscle cell differentiation medium comprises one or more components selected from Table I.

Regulation of Smooth Muscle Cell Differentiation by AT-Rich Interaction Domain Transcription Factors Mrf2α and Mrf2β

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Abstract—Despite the importance of vascular smooth muscle cells in the regulation of blood vessel function, the molecular mechanisms governing their development and differentiation remain poorly understood. Using an in vitro system whereby a pluripotent neural crest cell line (MONC-1) can be induced to differentiate into smooth muscle cells, we isolated a cDNA fragment that was robustly induced during this differentiation process. Sequence analysis revealed high homology to a partial cDNA termed modulator recognition factor 2 (Mrf2). Because the full-length cDNA has not been reported, we cloned the full-length Mrf2 cDNA by cDNA library screening and 5' rapid amplification of cDNA ends and identified two isoforms of Mrf2 (α [3.0 kb] and β [3.7 kb]) that differ in the N-terminus but share the DNA-binding domain. Protein homology analysis suggests that Mrf2 is a member of the AT-rich interaction domain family of transcription factors, which are known to be critically involved in the regulation of development and cellular differentiation. Mrf2α and Mrf2β are highly induced during in vitro differentiation of MONC-1 cells into smooth muscle cells, and Mrf2α is expressed in adult mouse cardiac and vascular tissues. To define the function of Mrf2, we overexpressed both isoforms in 3T3 fibroblast cells and observed an induction of smooth muscle marker genes, including smooth muscle α-actin and smooth muscle 22α. Furthermore, Mrf2α and Mrf2β retarded cellular proliferation. These data implicate Mrf2 as a novel regulator of smooth muscle cell differentiation and proliferation. (*Circ Res.* 2002;91:382-389.)

Key Words: smooth muscle ■ differentiation ■ proliferation ■ transcription factors

The vascular smooth muscle cell (SMC) is a critical cellular constituent of the blood vessel wall. In the adult blood vessel, vascular SMCs express a characteristic set of genes, exhibit a low proliferative index, and are involved in contraction and relaxation. In response to blood vessel wall injury, the vascular SMCs undergo a remarkable change in phenotype characterized by a decrease in contractile protein expression, reexpression of "fetal" smooth muscle (SM) genes, an increase in cellular proliferation, and an enhanced elaboration of extracellular matrix products.[1] These phenotypic changes are important in the pathogenesis of occlusive vascular disease evident in a number of clinical disease entities, such as arteriosclerosis, restenosis after percutaneous revascularization, and transplant vasculopathy.[1]

It has been suggested that this response to injury recapitulates a genetic program normally instituted during fetal development.[2,3] We reasoned that an understanding of normal mechanisms governing SM differentiation may allow one to modulate the SMC phenotype in disease states in a therapeutically beneficial manner. However, despite the importance of the SMC, the molecular mechanism(s) governing the physiological development of this cell and its response to pathological stimuli remain poorly understood. One of the principal reasons is the lack of an in vitro system for SMC differentiation. Previous studies have shown that primary mouse neural crest cells can be induced to differentiate into SMCs.[4] Furthermore, we reported an in vitro system whereby a pluripotent neural crest cell line (MONC-1) could be forced to assume the morphological and genetic characteristic of SMCs.[5] Differentiation can be initiated by a change in medium and results in the induction of SMC genes, such as SM α-actin, SM22α, calponin, APEG-1, and SM myosin heavy chain (SM1).

To identify factors that may regulate this process, we used differential mRNA display to identify transcripts that are differentially expressed in MONC-1 cells before and after SMC differentiation. We identified a cDNA fragment whose expression was induced <3 hours after the initiation of the

SMC differentiation. Sequence analysis revealed high homology to a partial cDNA termed the modulator recognition factor 2 (Mrf2) gene.[6,7] Because the full-length Mrf2 has not been reported, we cloned it and identified two isoforms. Protein homology analysis suggests that Mrf2 is a member of the AT-rich interaction domain (ARID) family of transcription factors.[8] In the present study, we provide evidence that Mrf2 may serve as a regulator of SMC differentiation and proliferation.

Materials and Methods

Cell Culture and Reagents

The mouse neural crest cell line, MONC-1,[9] was kindly provided by David Anderson (California Institute of Technology, Pasadena, Calif) and cultured as described.[5,10] SMC differentiation of MONC-1 cells was induced by applying medium 199 supplemented with 10% FBS (HyClone), 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 mmol/L HEPES (pH 7.4), hereafter referred to as SMC differentiation medium (SMDM). Differentiation down the neuronal and glial pathways was performed as described.[11] Rat aortic SM cells (RASMCs) were harvested from the thoracic aortas of adult male Sprague-Dawley rats (200 to 250 g; Zivic-Miller Co, Zelienople, Pa) by enzymatic digestion, according to the method of Gunther et al.[12] NIH-3T3 cells, A10 cells, and A7r5 cells were obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS. Human aortic SMCs (HASMCs) and human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics. 293T cells were obtained from Dr Hamid Band (New England Medical Center, Boston, Mass) and maintained as described.[13] Most of the media and the components of medium were obtained from Invitrogen. Animal care and procedures were approved by the Harvard Medical School Standing Committee on Animals and were performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care (NIH).

RNA Extraction and Northern Analysis

Total RNA from cultured cells and mouse tissue was prepared by guanidinium isothiocyanate extraction and centrifugation through cesium chloride[14] or by using an RNeasy RNA purification kit (Qiagen). Total RNA was fractionated on a 1.3% formaldehyde-agarose gel and transferred to nitrocellulose filters, which were hybridized with the appropriate [^{32}P]dCTP-labeled probe by a random-primed kit (Stratagene). The hybridized filters were washed in 30 mmol/L sodium chloride, 3 mmol/L sodium citrate, and 0.1% SDS at 55°C and exposed to Kodak XAR film at -80°C. To correct for differences in RNA loading, the blots were hybridized with an 18S rRNA oligonucleotide probe. To detect specific mRNA expression of each Mrf2 isoform by Northern analysis, we prepared an Mrf2α-specific probe in the 5' untranslated region of Mrf2α by polymerase chain reaction (PCR) with primers G9017 up (5'-CTGAGCCTCCTCGCAGCT-3') and G9017 low (5'-TTCCAGTCGCCAAACATCTG-3'). An Mrf2β-specific probe (431 bp) was obtained by *Bam*HI restriction digestion from the 5' coding region of Mrf2β. Mouse SM α-actin, mouse SM22α, mouse calponin, and mouse GAPDH were prepared by a RT-PCR technique.

Differential mRNA Display

We prepared several RNA samples from undifferentiated MONC-1 cells and MONC-1 cells cultured in SMDM for 10, 24, and 96 hours. Differential display analysis of these mRNA samples was performed using the HIEROGLYPH mRNA Profile Kit for Differential Display Analysis (Genomyx). The expression levels of these cDNA fragments were compared in duplicate on polyacrylamide gels. Each fragment of interest was isolated from gel, reamplified, and analyzed.

5'-RACE

The 5' end of mouse Mrf2 was cloned by the 5' rapid amplification of cDNA ends (5'-RACE) system (Invitrogen) using mouse aortic RNA. Primers used were as follows: G9008-1, 5'-TCCTTTGAGCCACTGA-3', for reverse transcription; G9008-2, 5'-GAGCCACTGAAGGAATCTCT-3', for the initial PCR; and G9008-3, 5'-CGCCATTCCAGTCGCCAAA-3', for the nested PCR.

Cloning of Full-Length Mouse Mrf2 by Library Screening

Differentiation display product DD101 was used to screen a mouse 11-day embryo 5' stretch plus cDNA library (Clontech) according to the manufacturer's protocol. Several clones were obtained, and a fragment from the most 3' cDNA clone was used to screen the library again. The sequences from these clones and 5'-RACE PCR product were assembled, and the full length of the Mrf2 isoforms was determined.

Computational Analysis

Sequence analysis was performed using Sequencher software (Gene Code Corp). Protein alignment and phylogenetic analysis were performed with the Clustal V algorithm included within the MegaAlign module of the Lasergene software package (DNASTAR).
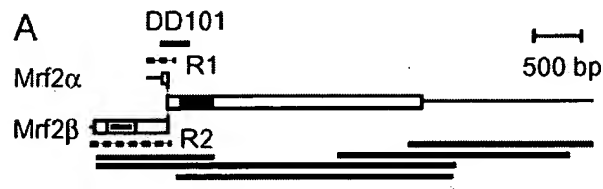
Cloning of Full-Length hMrf2 by Sequencing Est Clones and GenBank Search

A homology search with reported human Mrf2 (hMrf2) fragment in the human Est database of the National Center for Biotechnology Information and The Institute for Genomic Research was performed, and two Est cDNA clones (zm02a05.r1 and THC558757) overlapping the hMrf2 cDNA were found. After sequencing the clone, zm02a05.r1 (American Type Culture Collection), we assembled those sequences and generated the 3060-bp partial human cDNA, which included the stop codon. To clone the 5' end of hMrf2, we searched the human genomic sequence with the mouse Mrf2 sequence and identified two nonoverlapping genomic fragments (NT_008797.9 and NT_033984.1) containing the entire hMrf2 coding region. We found an additional 507-bp 5' end of the hMrf2 cDNA sequence including the ATG translation start site, and we assembled the putative full length of hMrf2 cDNA.

Construction of Plasmids

Mouse Mrf2 mammalian expression vectors were constructed by PCR in two steps. First, the 1.3-kb 5' portion of Mrf2α and the 2.1-kb portion of Mrf2β were amplified from MONC-1 RNA by RT-PCR using PfuTurbo DNA polymerase (Stratagene) with primers GK19 and GK24 and primers GK25 and GK24, respectively. The 1.5-kb 3' portion of common Mrf2 was amplified from MONC-1 RNA with primer GK23 and primer GK21. These amplified fragments were cloned into the pCR-BluntII TOPO vector (Invitrogen). Next, two parts of cloned fragments were combined and subcloned into the mammalian expression vector pcDNA3.1($-$) (Invitrogen). The primer sequences to construct these plasmids are as follows: GK19, 5'-GCTCTAGAGTTTGGCGACTGGAATGGCG-3'; GK21, 5'-GCCTCGAGGGTGCAATGGACCACACCATT-3'; GK23, 5'-TGA-GAACCACGGACTTAACTA-3'; GK24, 5'-ATAGGGATAGCTGCT-GGAGA-3'; and GK25, 5'-GCTCTAGAACGTCGAGATGGAGCC-3'.

To construct the expression plasmid Mrf2α-c-myc and Mrf2β-c-myc, we added in frame a DNA sequence encoding a c-Myc peptide tag (EQKLISEED) to the open reading frame at the 3' end of Mrf2α and Mrf2β-pcDNA3.1 by PCR techniques. We generated truncated Mrf2β(ΔC493)-c-myc-His and Mrf2α(ΔC493)-c-myc-His by subcloning the 5' 2.1-kb fragment of Mrf2β and the 5' 1.4-kb fragment of Mrf2α, respectively, into pcDNA3.1/Myc-His($-$) A. Both constructs lack 493 amino acids of the C-terminus. We also constructed truncated Mrf2β(ΔC493)-flag by adding in frame a DNA sequence encoding a flag tag to the 5' end of the open reading frame in pcDNA3.1. All plasmids were verified by sequencing.
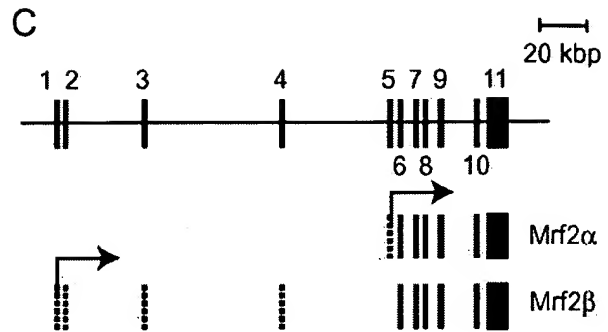
Figure 1. Cloning and protein sequence of mouse (M) and human (H) Mrf2. A, Nucleotide sequences of open reading frame of Mrf2α (2835 bp) and Mrf2β (3567 bp) were assembled from 5 phage cDNA clones (solid lines) and 5′-RACE products (R1 and R2, dashed lines). Original differential display fragment (DD101) is indicated by a solid line. The 2 isoforms differ at the 5′ end but are identical after Ala245 (indicated by asterisk in panel B). Protein motif searches identified an ARID (solid box in panel A and underlining in panel B) and a BAH-like domain (gray box in panel A and dashed underlining in panel B). B, The 1188-residue mouse Mrf2β and human Mrf2β proteins are aligned. The identical residue of human Mrf2 with mouse Mrf2 are indicated by dashes (−). Number sign indicates gaps, and a putative nuclear localization signal (RKKK) is indicated in bold. C, In the top panel, exons of mouse Mrf2 coding region are presented as black boxes (size of boxes may not always reflect size of exon) on the sequence of the mouse genomic clone (NW_000027.1). In bottom panel, usage of exons by Mrf2α and Mrf2β are shown. Dashed boxes represent isoform-specific exons, and a common region for both isoforms is indicated with black boxes.

Immunofluorescent Staining

NIH-3T3 cells grown on the glass slides were transiently transfected with the Mrf2β-myc–expressing plasmid using Fugene6 (Roche). Forty-eight hours after transfection, the cells were fixed, stained using an anti–c-Myc monoclonal antibody (9E10, Oncogene), and visualized with a fluorescence microscope as described.[15] Nuclei were counterstained with bis-benzimide (Molecular Probes).

Electrophoretic Mobility Shift Analysis

Nuclear extracts were prepared according to the method of Ritzenthaler et al[16] with minor modifications. Double-stranded oligonucleotide probes (5′-AGCTCCACAATACCAGGA-3′) synthe- sized according to the sequence of the Mrf2 core binding element (underlined)[6] were [^{32}P]ATP-labeled by T4 polynucleotide kinase (New England Biolabs). The reaction mixtures were incubated at 4°C for 3 hours in a total volume of 20 μL containing 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L KCl, 1 mmol/L DL-dithiothreitol, 5% glycerol, 0.5 μg of poly dI-dC (Amersham), 2.0×10⁴ cpm of labeled probe, and 20 μg of nuclear extract. The mixtures were analyzed by 4% native polyacrylamide gel electrophoresis in 0.25× TBE buffer (containing 22 mmol/L Tris base, 22 mmol/L boric acid, and 0.5 mmol/L EDTA) at 4°C. A 250-fold excess of specific or nonspecific oligonucleotide was used for competition experiments. The sequence of double-stranded oligonucleotide used as competitor was as follows: mutated Mrf2 core binding element, 5′-
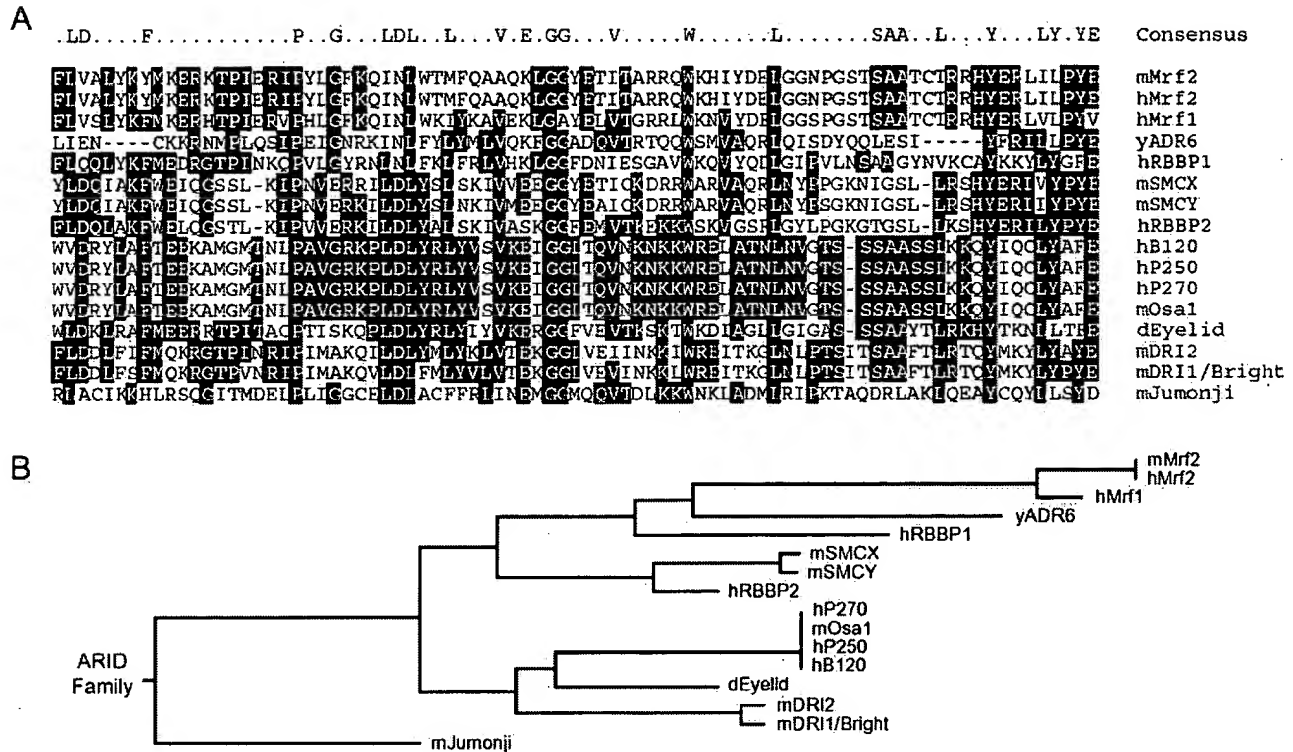
A

B

Figure 2. Phylogenetic analysis of Mrf2. A, Amino acids of the deduced ARID domain of several ARID family proteins were aligned by the Clustal V method. Amino acids conserved in >50% of the family members are shown (inverted). Prefix h indicates human; prefix m, mouse. B, Phylogenetic analysis, based on the ARID domain sequence of Mrf2 and other ARID family proteins by the Clustal V algorithm, was performed. The GenBank accession numbers for the sequences used in the these analysis are as follows: AA36325 hMrf1, P09547 yADR6, AF214114 hRBBP-1, AF127245 mSMCX, AF127244 mSMCY, NP_005047 hRBBP-2, CAB92121 hB120, AAG17549 hP250, AAF75765 hp270, AAK54504 mOsa1, AF053091 dEyelid, NP_062663 DRI2, NP_031906 mDRI1/Bright, and NP_068678 mJumonji.

AGCTCCACAcgACCAGGA-3'; CArG-binding element,[17] 5'-CTTTCCCCAAATATGGAGCCTG-3'; and nonspecific oligonucleotide, 5'-AGCTGTCAGCCCATGTGGCGTGGCCGCA-3'.

Retroviral Constructs and Transduction

The GFP-RV retrovirus vector and the Phoenix-Eco packaging cell line were obtained from Dr K. Murphy (Washington University School of Medicine, St. Louis, Mo).[18,19] Full-length Mrf2α and Mrf2β cDNA fragments amplified by PCR were subcloned into GFP-RV vectors. These expression vectors simultaneously express Mrf2 and GFP cDNA. Transfection of the packaging cell line and retroviral transductions were performed as described.[19] Infection of NIH-3T3 cells in the presence of 4 μg/mL hexadimethrine bromide (Polybrene) allowed for 100% infectivity.

Results

Identification of Mouse Mrf2α and Mrf2β

A differential display was used to identify cDNAs, which are regulated during SMC differentiation by using the MONC-1 system. Our criteria are as follows: (1) expression of the gene should be induced during MONC-1 cell differentiation, and (2) the gene should be also expressed in the adult mouse aorta. A number of cDNAs were isolated and sequenced, and their differential expressions were verified. A computer-assisted GenBank search revealed that one of the identified cDNAs (Figure 1A, DD101) was the mouse homologue of a partial human cDNA Mrf2 (hMrf2).[7] Although the full-length

cDNA of hMrf2 had not been reported at that time, binding site selection studies and crystal structure analysis using the DNA-binding domain revealed that this factor was able to bind to the A/T-rich consensus sequence AATA(C/T).[6,7] We used a combination of 5'-RACE and cDNA library screening to clone the full-length mouse Mrf2 cDNA (Figure 1A). These studies revealed two forms, which differ at the 5' end but are identical from Ala245 (indicated by asterisk in Figure 1B) to the C-terminus. The shorter product is termed Mrf2α, whose open reading frame is 2835 bp, with a predicted protein size of 104 kDa. The longer product, termed Mrf2β, is a 3564-bp open reading frame with a predicted size of 131 kDa.

Homology and Genetic Analysis of Mrf2

Using the mouse Mrf2 cDNA as a template, we assembled a putative cDNA sequence of hMrf2 from human Est clones, a 5'-RACE product, and the human genomic sequence. The amino acid and cDNA nucleotide sequences were well conserved between mouse and human (amino acid, 87% identity; nucleotide, 85%). We also identified an 83-amino-acid domain (underlining in Figure 1B) that has high homology to ARID[8] and another 73-amino-acid domain (dashed underlining in Figure 1B) in Mrf2β, which has a homology to the bromo-adjacent homology (BAH)

domain.[20] The sequence of the ARID domain was completely conserved between mice and humans. Examination of the mouse and human genome database reveals that there is one mouse clone (NW_000027.1) that contains all the coding region of mouse Mrf2 and two nonoverlapped clones (NT_008797.9 and NT_033984.1) that contain all the coding region of hMrf2. In both species, these clones are located in chromosome 10. We deduced the genomic structure of both mouse Mrf2 and hMrf2 from these clone sequences in GenBank and found that both species have almost identical genomic structures with 11 exons (Figure 1C). Exons 5 to 11 constitute Mrf2α cDNA, whereas exons 1 to 4 and 6 to 11 constitute Mrf2β (Figure 1C). Finally, sequence alignment and phylogenetic analyses suggest that Mrf1 and Mrf2 form a distinct subclass of the ARID family of transcription factors (Figures 2A and 2B).

Cellular and Tissue Expression of Mrf2

Mrf2 was identified on the basis of its differential expression in undifferentiated versus differentiated MONC-1 cells. We verified this by Northern blot analysis using cDNA fragments specific to Mrf2α and Mrf2β. Both Mrf2α and Mrf2β were induced during MONC-1 differentiation (Figure 3A). The expression of Mrf2α is more restricted because induction was not observed when MONC-1 cells were differentiated down the neuronal pathway. Both isoforms were also expressed in cultured primary RASMCs and HASMCs (Figure 3B) and the SMC line A7r5 but not in HUVECs. To determine the tissue expression of Mrf2 in adult mice, we isolated total RNA from a number of adult mouse tissues and subjected them to Northern analysis by using the isoform-specific probes. These studies revealed distinct expression patterns for the two isoforms. Mrf2α mRNA was detected in the aorta, heart, and lung. In contrast, low Mrf2β mRNA expression was detected in the lung and spleen, and very low expression was detected in the aorta and kidney (Figure 3C).

Mrf2 Is a Nuclear Protein and Binds to A/T-Rich Sequences

Analysis of the Mrf2 cDNA revealed the presence of a potential nuclear localization signal in the region shared by Mrf2α and Mrf2β (Figure 1B, bold). To assess cellular localization, we cloned the full length of both isoforms bearing a C-terminal c-myc tag into a mammalian expression vector and transiently transfected into NIH-3T3 cells. Immunocytochemical analysis using an anti-myc antibody revealed that Mrf2α showed only nuclear localization (Figure 4A). A similar nuclear localization pattern was observed with Mrf2β (data not shown).

The 108-amino-acid ARID domain of Mrf2 is shown to bind AATA(C/T) as a consensus binding site.[6] This is of interest in SM biology, because A/T-rich sequences, termed CArG boxes, have been implicated as being critical in the regulation of SMC genes, such as SM α-actin,[21] SM22α,[17] calponin,[22] and the SM1[23] promoters. To assess the ability of Mrf2 to bind these sequences, we performed electrophoretic mobility shift assays using nuclear extracts from 293T cells transfected with several Mrf2 constructs. We did not observe any specific retarded bands in the

presence of full-length Mrf2α and Mrf2β. However, C-terminal truncations of both isoforms, Mrf2β(ΔC493) and Mrf2α(ΔC493), which retain the ARID DNA-binding domain, were able to bind to the Mrf2-binding site. To verify the specificity of this band, competition and supershift studies were performed. As shown in Figure 4C, the binding of Mrf2β(ΔC493) could be competed away with an identical competitor probe but not a mutated oligomer.
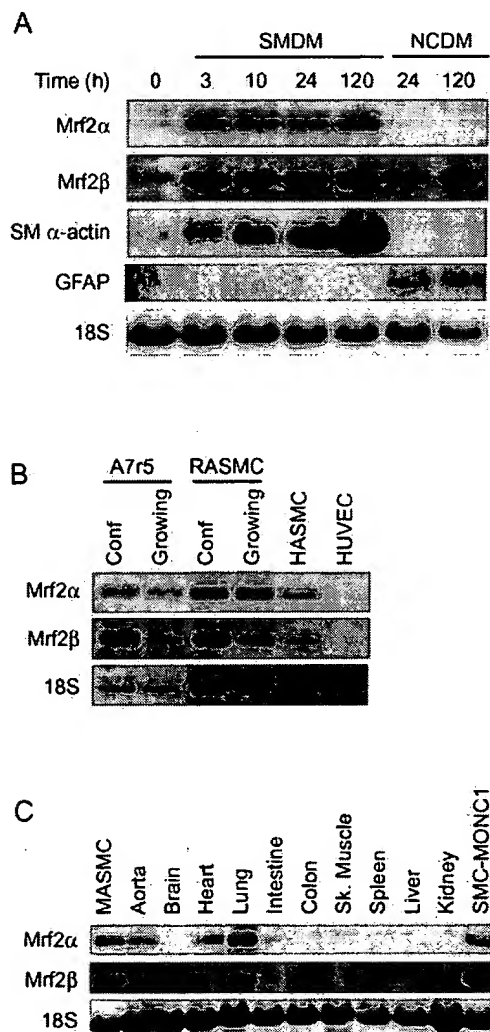

Figure 3. Cellular and tissue distribution of Mrf2 by Northern analysis. Total RNA (10 μg) was prepared from MONC-1 cells (A), cultured cell lines or primary cells (B), and adult mouse tissues (C) and separated by gel electrophoresis. Northern analysis was performed by hybridizing blots sequentially with the indicated probes as described in Materials and Methods. A, Total RNA was prepared from undifferentiated MONC-1 at 0, 3, 10, 24, and 120 hours after the initiation of SMC differentiation by SMDM and at 24 and 120 hours after the initiation of neuronal cell differentiation by neuronal cell differentiation medium (NCDM). Expression of SM α-actin was shown to demonstrate SM differentiation, and expression of glial fibrillary acidic protein (GFAP) was presented to show neural/glial cell differentiation by NCDM. B, Total RNA was prepared from confluent (Conf) and growing A7r5 and SMC lines and confluent and growing primary RASMCs, HASMCs, and HUVECs. C, RNA was prepared from adult mouse tissues, primary cultured mouse aortic SM cells (MASMCs), and SMC-differentiated MONC-1 cells (SMC-MONC-1).
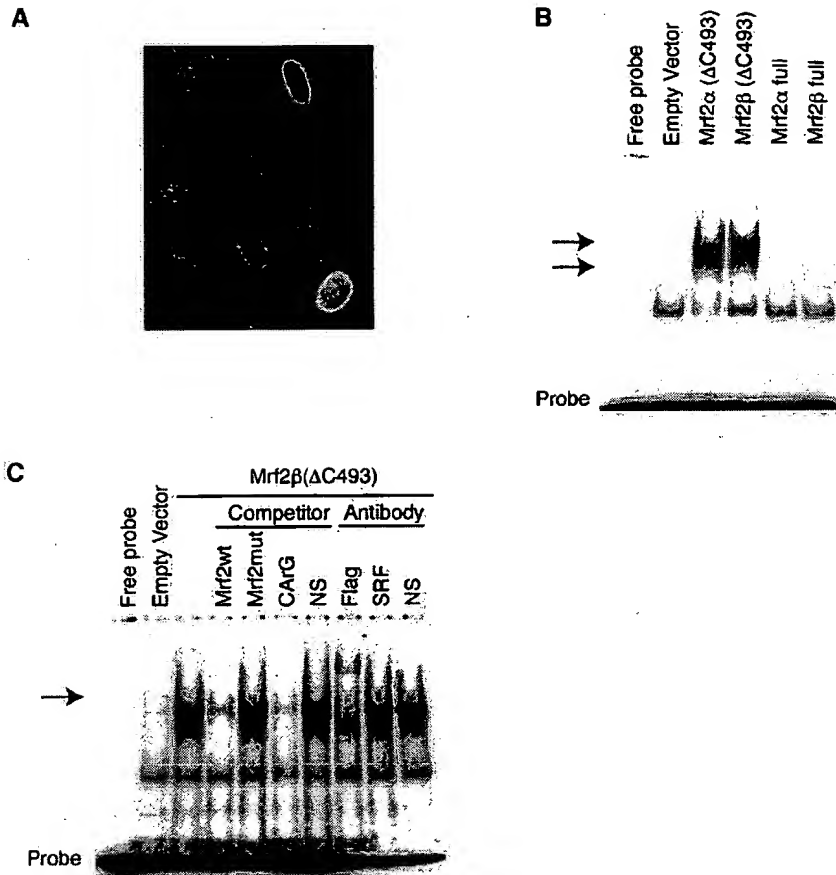
A

B

Probe

C

Probe

Figure 4. Cellular localization and DNA binding activity of Mrf2. A, NIH-3T3 cells were transiently transfected with the Mrf2α-c-myc mammalian expression vector. Forty-eight hours after transfection, cells were fixed and visualized with a fluorescence microscope as described in Materials and Methods. Nuclei were counterstained with bis-benzimide. B, Gel mobility shift assays were performed as described in Materials and Methods with a labeled probe containing the Mrf2 core-binding sequence by using nuclear extracts from 293T cells transfected by the empty vector, Mrf2α(ΔC493), Mrf2β(ΔC493), and Mrf2α full-length and Mrf2β full-length constructs. Upper arrows indicate the DNA and Mrf2β(ΔC493) complex (fourth lane), whereas the lower arrow pointed to the DNA and Mrf2α(ΔC493) complex (third lane). C, Gel mobility shift assays were performed in the same way as described for panel B. No specific binding activity was detected in nuclear extract transfected with empty pcDNA3.1 vectors (second lane). In other lanes, the nuclear extract from 293T cells transfected with Mrf2β(ΔC493) was used for analysis. A 250-fold excess of specific or nonspecific oligonucleotide was used for competitive binding experiments. The cold probe with nuclear factor-κB binding sequence was used as a nonspecific competitor. One microliter of antiserum to Flag, serum response factor (SRF), and c-myc (NS indicates nonspecific antiserum) was incubated with nuclear extracts to show the supershift of the DNA and protein complexes. The DNA-protein complex is indicated by an arrow.

Interestingly, an oligomer containing the CArG box was also able to compete for binding, suggesting that the DNA-binding domain of Mrf2 may bind to the CArG element (Figure 4C). This DNA-protein complex could be supershifted in the presence of an anti-Flag antibody but not an anti–serum response factor or nonspecific antibody (anti–c-myc). Identical results were seen with Mrf2α. These data suggest that Mrf2α and Mrf2β can bind to an A/T-rich consensus sequence.

Overexpression of Mrf2 Induces SMC Gene Expression

The induction of Mrf2 isoforms with MONC-1 differentiation raised the possibility that these factors may induce SMC marker genes. To test this hypothesis, we assessed the ability of both Mrf2α and Mrf2β (Figure 5) to regulate the expression of SM markers in a heterologous fibroblast cell line, NIH-3T3. Cells infected with empty virus (control) or uninfected cells were used as controls. We did not observe any evidence of toxicity from overexpression of any of these constructs. In contrast to control, overexpression of either Mrf2α or Mrf2β induced the expression of SMC genes, such as SM α-actin and SM22α (Figure 5). We did not observe any effect on the expression of calponin (Figure 5) or SM1 (data not shown). These data suggest that both Mrf2 isoforms can induce certain SMC genes, such as SM α-actin and SM22α.

Overexpression of Mrf2 Retards Cell Proliferation

Cellular differentiation is characteristically accompanied by a reduction in cellular proliferation. To assess whether Mrf2

can regulate cellular proliferation, 3T3 fibroblast cells were retrovirally infected, and the number of cells was counted on 2 days and 4 days after infection. We did not observe any evidence of cellular toxicity. Representative experimental
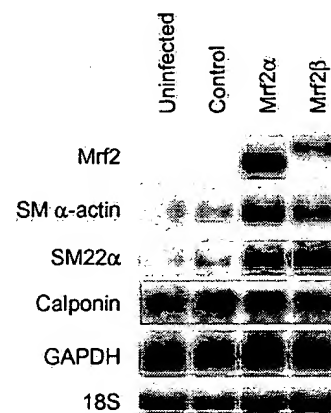

Figure 5. Mrf2 induces SMC marker gene expression. Mrf2α and Mrf2β were subcloned into GFP-RV vectors. Transfection of the retroviral packaging cell line and retroviral transductions were performed as described in Materials and Methods in a heterologous fibroblast cell line, NIH-3T3. After infection, 3T3 cells were cultured in DMEM supplemented with 0.4% calf serum for 48 hours, and 10 μg total RNA was prepared. After electrophoresis, RNA was transferred to nitrocellulose filters and hybridized with a ^{32}P-labeled common Mrf2 probe, SM α-actin probe, SM22α probe, calponin probe, and GAPDH probe. Filters were hybridized with 18S rRNA oligonucleotide probe to verify loading.
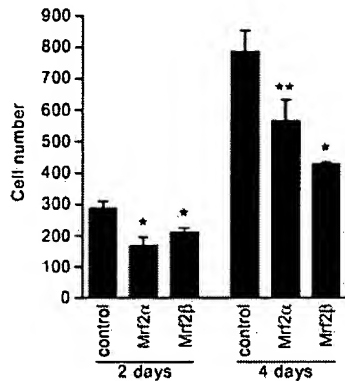
Figure 6. Mrf2 retards cell proliferation of 3T3 fibroblast cells. Fifty thousand 3T3 fibroblast cells were plated onto a well of 6-well plates. After retroviral infection, 3T3 cells were cultured for 2 or 4 days, and the number of cells was counted. Representative experiment is shown (mean±SD, n=3), and similar results were observed in 3 independent experiments. *P<0.01 and **P<0.02 vs empty vector.

data are shown (Figure 6), and similar results were observed in three independent experiments. Cells overexpressing either Mrf2α or Mrf2β exhibited an ≈30% to 40% reduction in cell number compared with empty vector (Figure 6). To exclude the possibility that the reduction in cellular proliferation was due to apoptosis, we tested for caspase-3 activity. No statistically significant difference was observed between uninfected cells, empty virus–infected cells, and Mrf2α and Mrf2β retrovirus–infected cells. In contrast, treatment of cells with staurosporine resulted in a significant apoptotic effect[24] (data not shown). Taken together, these data suggest that both Mrf2 isoforms may induce a differentiated and quiescent state of SMCs.

Discussion

Using differential display, we identified Mrf2 as a nuclear factor induced during MONC-1 cell differentiation into SMCs. Although a partial cDNA of Mrf2 had been previously published,[6,7,25] the full-length cDNA had not been reported. In the present study, we identified two isoforms of Mrf2 that differ by 245 amino acids at the 5' end. We also found that overexpression of both isoforms potently induces the expression of SMC markers and retards cellular proliferation.

A GenBank search revealed that Mrf2 has a high homology to a cDNA termed DESRT (accession No. AF169968).[26,27] Interestingly, the DESRT cDNA terminates at base pair 2178 because of a single nucleotide insertion, which results in a frame shift. Given that the predicted amino acids of DESRT and Mrf2 are similar, it is likely that they are very closely related gene or splice variants. The expression patterns of both Mrf2 isoforms are distinct, suggesting possible tissue-specific roles. With respect to SMCs, both isoforms are induced with differentiation. However, expression of the α isoform is induced only during SMC differentiation but not after neuronal cell differentiation. Furthermore, this isoform is strongly expressed in the aorta, suggesting that it is likely the dominant isoform in SMCs.

The DNA-binding domain of Mrf2 bears high homology to the ARID family of transcription factors (Figures 2A and 2B).

Nuclear magnetic resonance studies showed that the structure of the Mrf2 domain is significantly different from the known DNA-binding domain and consists of helices and loops but not any β strands.[7] ARID is shared by several proteins in a variety of organisms, such as yeast, plants, Drosophila, and vertebrates.[8] Mrf2 does not contain jmjN or jmjC motifs[28] or REKLES domains,[7] which are shared by other members of the ARID family protein. Although the full length of Mrf1 is not reported, Mrf1 and Mrf2 may constitute a distinct subgroup of ARID proteins (Figure 2B). The specific roles of the ARID domain have not been fully elucidated, but ARID family proteins are involved in a number of biological processes, including embryonic development,[29] cell lineage gene regulation,[30] and cell cycle control.[31] We also identified in Mrf2β a region bearing homology to the BAH domain, which has been implicated in chromatin regulation.[20] Because ARID family proteins are repeatedly suggested to be involved in chromatin regulation,[30,32,33] some aspect of Mrf2 function may involve chromatin modification and regulation.

In most biological systems, cellular differentiation is accompanied by a reduction in cellular growth rates. The induction of Mrf2 isoforms during SMC differentiation raised the possibility of a role in cellular differentiation. To formally address this possibility, we overexpressed both Mrf2 isoforms and assessed the effects on the expression of SMC differentiation markers and cellular proliferation. Indeed, we observed that overexpression of both Mrf2 isoforms induced the expression of certain classical SMC markers and retarded cellular proliferation (Figures 5 and 6). Specifically, we observed that Mrf2 overexpression induced SM α-actin and SM22α but not other markers, such as calponin, SM1, or smoothelin (Figure 5 and data not shown). The expressions of SM α-actin and SM22α are early markers of the SMC lineage but can also been seen in the conversion of fibroblasts into myofibroblasts. As such, Mrf2 may be involved in the early stages of differentiation into SMCs and/or into myofibroblasts. The induction of Mrf2 within hours of culturing MONC-1 cells in SM differentiation medium supports a role for these factors in the early stages of differentiation into SMCs. Previous studies highlight the importance of a *cis*-acting element, termed the CArG box, in the regulation of most SM genes, such as SM α-actin,[21] SM22α,[17] calponin,[22] and the SM1[23] promoters. We found that the CArG box oligomer derived from the SM22α promoter competed away Mrf2 binding in gel-shift studies, suggesting that Mrf2 may bind to this element (Figure 4B). However, we were unable to show direct Mrf2 binding to the CArG box in gel-mobility shift assays using in vitro–transcribed and in vitro–translated products or recombinant protein, thereby supporting a non-CArG-dependent mechanism. Alternatively, it is still possible that Mrf2 may bind the CArG element in vivo in the presence of critical cofactors or in the appropriate DNA context. These issues are the focus of ongoing studies.

been deposited in the GenBank with accession Nos. AF401550 and AF280065, respectively.

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